"Antitumor effect of *Trigonella foenum graecum* Seed Extract on skin papillomagenesis in Swiss Albino Mice."

Submitted

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INTRODUCTION:

Cancer is a disease as characterized by the uncontrolled proliferation of cells. It results from a breakdown of the regulatory mechanisms that govern the division, differentiation and survival of individual cells. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs. Out of many known cancer this study primarily focuses on the tumors caused in the skin.

Skin is a shield that protects people from heat or cold, chemicals, UV-radiation and bacteria. Skin cancer is one of the most common of all human cancers and its incidence is increasing rapidly all over the world. Approximately 30% of all newly diagnosed cancer in the world is skin cancer (Manoharan *et al*., 2010). There has been a progressive increase in the incidence of skin cancers, particularly that of cutaneous melanomas over the last few decades. (Howe *et al.,*2001). In India skin cancers constitute about 1-2% of all diagnosed cancers. Various studies from India have consistently reported SCC (squamous cell carcinoma) as the most prevalent skin malignancy (Godbole *et al.,* 1968). Owing to the large population the incidence of skin cancer in India is lower as compared to the western world. This fact has been established by Deo *et al,* in 2005.

Epidemiological studies have shown that 70-90% of all cancers are environmental (Sharma *et al*., 2010).Also lifestyle related factors are the most important and preventable among the environmental exposures. The risk factors of the major non-communicable diseases like cancer are tobacco, dietary habits, inadequate physical activity and alcohol consumption (Oliviera *et al*., 2007). Carcinogens in tobacco smoke are classified as human carcinogens with sufficient evidence. These carcinogens include **polyaromatic hydrocarbons (PAH)**, heterocyclic hydrocarbons, N-nitrosamines, aromatic amines, aldehydes, inorganic compounds, and radio-elements such as polonium (Sophia *et al*., 1986). Benzo(a)pyrene (BaP) and **7,12-dimethylbenz(a)anthracene (DMBA)** are polycyclic aromatic hydrocarbons (PAHs) found in the tar fraction of cigarette smoke, as well as in car exhaust(Lee *et al.,* 2002). Tumor induction studies with many PAH, including dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P), 7,12-dimethylbenz[*a*]anthracene (DMBA), and benzo[*a*]pyrene (B[*a*]P) have been studied. It was demonstrated that DMBA is a significantly stronger tumor initiator than B[*a*]P tested in mouse skin and rat mammary gland.

MULTISTAGE SKIN CARCINOGENESIS

The development of cancer is a complex process during which a normal cell undergoes a progressive series of alterations resulting in the acquisition of an altered proliferative capacity, invasiveness and metastatic potential. These alterations occur in 3 stages: **initiation** which involves DNA damage leading to mutation(s); followed by **promotion**, which involves enhanced proliferation and altered cell behavior; and finally **progression** results from subsequent genetic changes such as loss of heterozygosity and gene amplification.

The present work titled "Antitumor effect of *Trigonella foenum graecum* on two stage skin pappilomagenesis in Swiss Albino Mice" aims to deal with the role of these two carcinogens DMBA and TPA in the induction of skin carcinogenesis. Furthermore the study covers the phytoremediation in cancer treatment with the efficacy of *Trigonella*.

DMBA:

Since the emphasis of the report is on the DMBA,a polycyclic aromatic hydrocarbon(PAH), studies have shown that animals treated with DMBA showed 100% tumor incidence and the tumor was histopathologically confirmed as well differentiated squamous cell carcinoma. It reflects a close relationship between dimethylbenz(a)anthracene and skin carcinogenesis. However DMBA is also responsible for induction of mammary tumors in winstar rats (Margarida *et al.,* 2010).To understand the complex process of carcinogenesis by PAHs sound knowledge is required.

7, 12-Dimethylbenz(a)anthracene is an immunosuppressor and a powerful organ-specific laboratory carcinogen. DMBA serves as a tumor initiator by making necessary mutations.

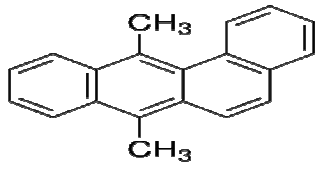


Fig: DMBA

TPA:

During the development of squamous malignancy the first stage i.e. initiation is followed by promotion, the second stage. Promotion involves repeated exposure to an agent that permits and increases the development of benign tumours, or papillomas, in an initiated cell population. Some of the most commonly studied types of tumour promoters in the mouse-skin carcinogenesis model belong to the phorbol-ester family: diterpenes originally isolated from the seed oil of the plant *Croton tiglium*.

One of the most potent and widely used phorbol esters is phorbol 12-myristate 13-acetate or tetradecanoyl phorbol acetate (PMA or TPA)(Griner *et al.,* 2007). It is very soluble in most polar organic solvents, as well as in water and mimic diacyl glycerols.

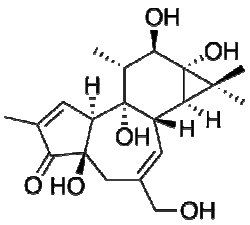


Fig: TPA

PLANT PRODUCTS AS MEDICINES

The use of natural remedies for the treatment of liver and kidney diseases has a long history and medicinal plants and their derivatives are still used all over the world in one form or the other for this purpose.

Ayurveda with its vast modalities is an integral spiritual science, which is gifted to the universe from the ancient enlightened Vedic culture. This science of life, which is emerging as the global medicine accords a high place for the dark green leafy vegetables in the daily diet. India, with its enormous natural resources, is bestowed with thousands of varieties of leafy vegetables, which is highly energy giving. From time immortal, man depended on plants as medicine. These remedies are most efficacious as they are taken directly from nature. They are amazingly nutritious and powerhouse of antioxidants.

Fenugreek, *Trigonella Foenum-groecum* Linn , is an annual herb indigenous to the countries bordering on the eastern shores of the Mediterranean and largely cultivated in India, Egypt, and Morocco (Snehlata *et al.,* 2012).It belongs to the family Fabaceae. The alkaloid trigonelline, trigocoumarin, trimethyl coumarin and nicotinic acid are present in stem. The leaves contain 7 saponins, known as graecunins. Mature seeds content amino acid, fatty acid, vitamins, and saponins. The seeds of fenugreek contain a large quantity of folic acid (84mg/100g)(Toppo *et al.,*2009). It also contents disogenin, gitogenin, neogitogenin, homorientin saponaretin, neogigogenin, and trigogenin (Rastogi *et al.,*1990). The plants leaves and seeds are widely consumed as a spice in food preparations, and as an ingredient in traditional medicine (Syeda *et al.,*2008). Medicinally it was used for the treatment of wounds, abscesses, arthritis, bronchitis, ulcer and digestive problems. Apart from having few health benefits it has many pharmacological properties such as: antidiabetic activity, antibacterial activity, anti-inflammatory and analgesic activity, antioxidant activity.

REVIEW OF LITREATURE:

Skin cancer, the most common form of human cancer, proceeds through three distinct phases, initiation, promotion and progression. It represents the most commonly diagnosed cancer, surpassing lung, breasts, colorectal and prostate cancer. They start as precancerous lesions and environmental toxins play a crucial role in the initiation of skin carcinogenesis (Krueger *et al*., 2008; Boukamp, 2005). It also represents a major and growing public health problem and of all new cancers diagnosed annually in the world, almost one-third originates in the skin (Greenlee *et al.*, 2001). In India, skin cancer accounts for 1-2% of all cancers (Deo *et al.*, 2005).

7,12-dimethylbenz(a)anthracene **(DMBA)**, a polycyclic a polycyclic aromatic hydrocarbon, is a procarcinogen and thus needs metabolic activation to become an ultimate carcinogen (Miyata *et al*., 2001). The active metabolite, dihydrodiol epoxide, generated during the metabolic activation of DMBA binds to and causes damage to DNA. Excessive reactive oxygen species are also generated during metabolic activation of DMBA. It is widely used as an initiator to induce skin carcinogenesis in Swiss albino mice (Das and Bhattacharya, 2004; Nigam *et al.*, 2007). DMBA induces skin carcinogenesis and is therefore commonly employed to study the chemopreventive potential of natural and synthetic entities (Rastogi *et al*., 2007).

The mouse skin model has been used for decades as a reliable and conventional model for studying the mechanisms of carcinogenesis and modulation of sequential steps involved in the process. In skin, the epidermis is the main target for various initiators and promoters. Skin tumors can be induced by the sequential application of a sub-threshold dose of a carcinogen (initiation stage) followed by repetitive treatment with a non-carcinogenic promoter (Yuspa, 1994). Topical application of **TPA**, the classical tumor promoter to mouse skin, has been shown to generate a number of biochemical alterations, changes in cellular functions, and histological changes leading to skin tumor promotion. Example of alterations are: epidermal cell proliferation, recruits inflammatory cells, increases production of reactive oxygen species (ROS) leading to oxidative DNA damage, and reduces DNA repairing capability.

Oxidative stress occurs in the cells due to imbalance in oxidant and antioxidant status. Oxidative stress damages DNA, lipids and proteins as well impair the structure and function of biomembranes (Droge, 2002). Reactive oxygen species (ROS) mediated lipid peroxidation has been implicated in the pathogenesis of several cancers including skin cancer. Mammalian cells have, however, an array of sophisticated antioxidant defense mechanism to compromise and combat the deleterious effects of ROS. Though skin antioxidants maintain the balance of cellular redox balance, premature ageing of skin and tumor initiation occurs if ROS are excessively generated in the skin (Lu *et al*., 2007; Ishii, 2007).

Chemoprevention, a novel and appealing strategy, deals with the inhibition, reversal or suppression of carcinogenesis by the use of natural or synthetic agents (F'guyer *et al*., 2003). The possible mechanism so far reported for the chemopreventive potential of natural products include carcinogen detoxification, suppression of genetic mutation, suppression of cell proliferation, induction of apoptosis and modulation of the immune system (Dorai and Aggarwal, 2004). Agents that possess antimutagenic and antioxidant potential have the ability to exert striking inhibitory effects on diverse cellular events associated with multistage carcinogenesis (Crowell, 2005).

Trigonella foenum graecum belonging to the family Papilionaceae commonly known as Fenugreek is an aromatic, 30-60 cm tall, annual herb. Fenugreek seeds are a rich source of the polysaccharide galactomannan, of saponins such as diosgenin, yamogenin, gitogenin, tigogenin, and neotigogens.flavonoids and amino acid,alkaloids, Other bioactive constituents of fenugreek include mucilage, volatile oils.

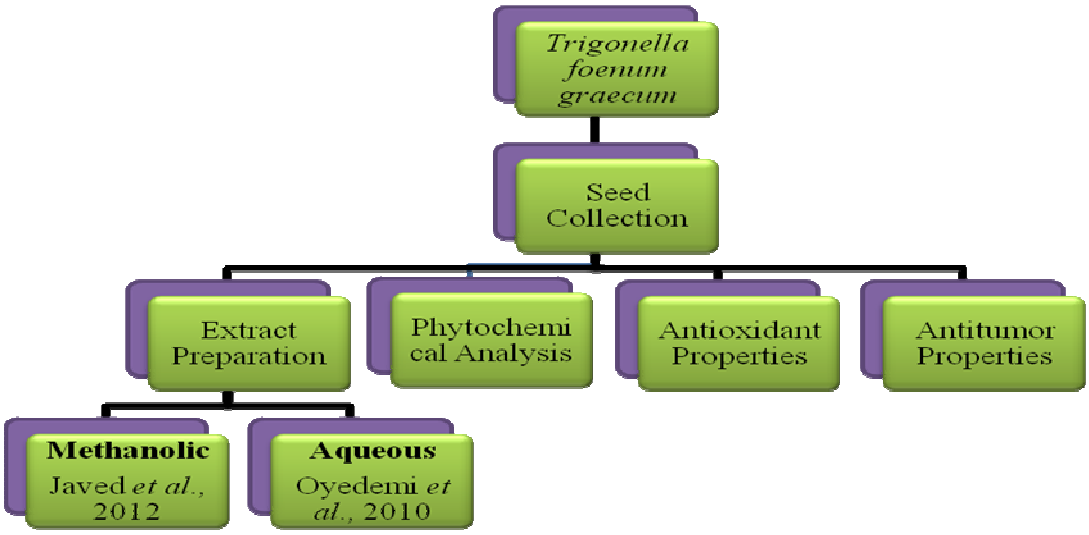
Experimental studies demonstrated several pharmacological activities including antioxidant and anticancer potential of *Trigonella foenum graceum.* Fenugreek seed extract has been shown to improve intraperitoneal glucose tolerance in normal mice. The aqueous seed extracts of Fenugreek were found more effective against *E. coli*, *S.typhi* and *S aureus*. Fenugreek seeds' extract significantly inhibited the DMBA-induced mammary hyperplasia and decreased its incidence (Yadav *et al.,* 2011). The present study is designed to evaluate the antitumor effect of *Trigonella foenum graceum* on skin papillomagenesis in Swiss Albino Mice.

OBJECTIVES:

1. To evaluate the tumirogenecity of the two commonly used carcinogens DMBA and TPA.
2. To screen the plant (*T.foenum graecum*) for its phytochemical properties and examine its antioxidant properties.
3. To evaluate the antitumor and antioxidant effect of *Trigonella foenum graecum* seed extract on mice.

METHODOLOGY AND PLAN OF WORK:

In this proposed plan antitumor role of (*Trigonella foenum-graecum/* Fenugreek/Methi seeds) will be studied in Swiss Albino mice which will be exposed to two carcinogens DMBA and TPA.



A1) PREPARATION OF EXTRACT:

***Trigonella foenum*:** Seeds will be selected for study. The seeds will be shade dried overnight. The dried seeds will then be grounded into powder form and can be stored at 4°C until further use. The extracts which will be prepared are: **Methanolic and Aqueous extract.**

A2) PHYTOCHEMICAL ANALYSIS:

After the extract preparation various tests will be performed to screen different macromolecules like:

NAME OF THE MACROMOLECULE	NAME OF THE TEST	REFERENCES
Carbohydrates	Molisch's test, Barfoed's Test	Sofowara., 1993
Alkaloids	Mayer's test Wagner's test Hager's test	Evans, 1997 Wagner, 1993 Wagner *et al.,*1996
Saponins	Froth test, Foam test	Kokate., 1999
Phytosterols	Liebemann Buchard's test, Salkowski's test	Finar., 1986
Phenols	Ferric Chloride Test	Mace, 1963
Tannins	Gelatin test, General test	Evans, 1997
Fats &Fixed Oils	Spot test, Saponification test	Kokate, 1999
Glycosides	Lega's test, Kelle-killani test	Evans, 1997
Flavanoids	Alkaline Reagent Test Shinoda Test	Trease and Evans, 2002 Harborne, 1998

A3) ANTIOXIDANT PROPERTIES

Free radicals are chemical entities that can exist separately with one or more unpaired electrons produced from various biochemical reactions. They are generated as a result of imbalance between formation and neutralization of pro-oxidants in the body metabolic process. Examples of these are reactive oxygen species (ROS) or reactive nitrogen species (RNS) radicals which include superoxide anions, singlet oxygen, hydrogen peroxide and hydroxyl radicals. There has been a global trend toward the use of natural substances present in medicinal plants as therapeutic antioxidant agents. They contain bioactive chemicals such as phenols, alkaloids and lignin which are potent radical terminators that can help in reducing the risk of cancer, toxicity, inflammation and cardiovascular diseases. Therefore, in the present study antioxidant activities of the seed extract of *T. foenum graecum* will be determined.

Antioxidant activity will be determined by:

NAME OF THE ASSAY	References
DPPH(1,1-diphenyl--2--picrylhydrazyl)	Vilasrao *et al.,* 2010
ABTS 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)	Re *et al.,* 1999
H_2O_2 (Hydrogen peroxide radical scavenging activity)	Ruch *et al.,* 1989
Nitric oxide scavenging activity	Garrat, 1964
Linoleic acid bleaching assay.	Miraliakbari & Shahidi., 2008

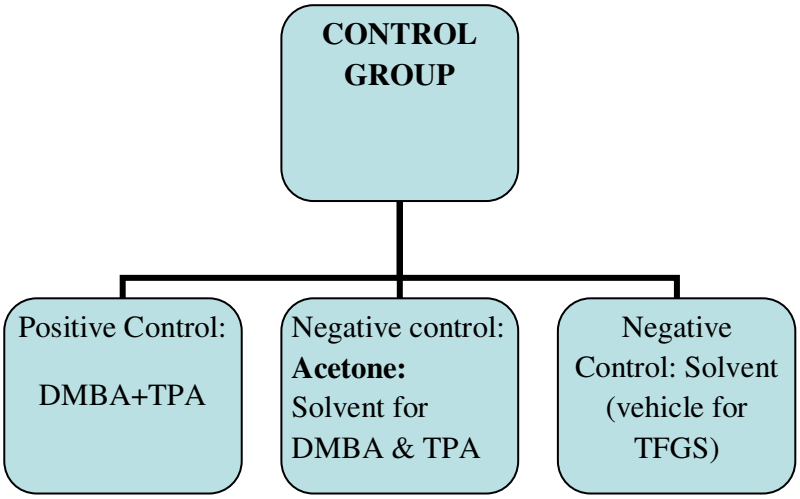
B.Experimental protocol

Maintenance of Animals: 50 Male Swiss Albino mice 6-8 weeks old will be maintained in standard condition: 23±2°C.

Relative Humidity: 55±10%.12:12 h LD Cycle and allowed free access to food and water. They will be housed in separate polypropylene cages containing sterile paddy husk as bedding material.

Animals will be divided into two groups namely: control and treatment group which will be further subdivided into subgroups of 7 each.

Group I: CONTROL GROUP


CONTROL GROUP
Positive Control: DMBA+TPA
Negative control: Acetone: Solvent for DMBA & TPA
Negative Control: Solvent (vehicle for TFGS)

Positive Control: DMBA+TPA

For the induction of tumors/papillomas, the two stage protocol consisting of initiation with single topical application of the carcinogen DMBA on the shaven back of mice, followed by three times a week treatment with a promoter TPA will be applied. TPA application on the skin will be for 14 weeks. Tumorigenicity of selected carcinogens DMBA and TPA will be studied at a particular dose level.

Negative Control: Acetone (Solvent for DMBA & TPA)

To observe the effects of acetone on mouse skin, acetone will be applied topically continuously for 16weeks.

Negative Control: Solvent system (Vehicle for TFGS)

A solvent will be taken as a vehicle for *Trigonella foenum graecum* and mice under this group will be orally given the solvent for 4 months.

DOSE TOLERANCE STUDY

Swiss Albino Mice will be divided into two groups: **Group A as control group and Group B as treatment group.** Group II mice will be further divided into six groups of seven mice each. Group I will administered vehicle only (any solvent) for seven days. Group II will be orally administered for seven days at a wide range of dose level by TFGS in a vehicle (solvent). After observing animals for consecutive seven days for morbidity, mortality and behavioural changes optimum dose will be selected. Experiment will also be conducted to test the influence of TFGS extract on mice liver LPO level and GSH level.

Group II: <u>TREATMENT GROUP</u>

Experiments will be designed to see the effect of TFGS extract on DMBA/TPA induced skin papillomagenesis.TFGS at an optimum dose will be administered both orally and topically as shown in the figure. At the end of experimental period, all the animals will be sacrificed by cervical dislocation. Liver will be blotted dry, weighed and processed for biochemical assays.

Bone marrow cells will be harvested and processed for chromosomal aberration assay and micronuclei assay. At molecular level transcription factor p53 will be evaluated either by Western Blotting or by PCR.

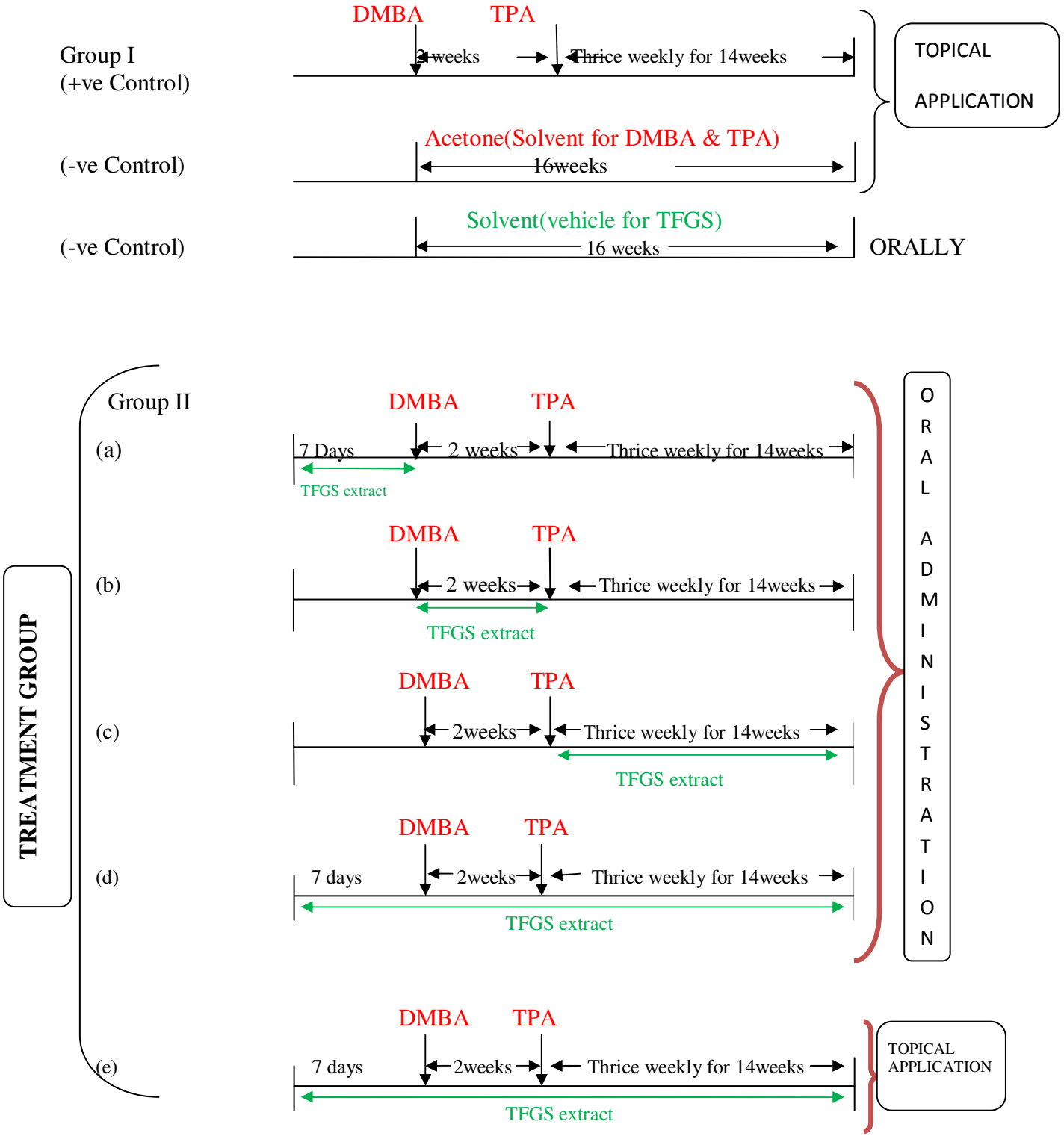


Fig: Dose application pattern in control and treatment group

C. PARAMETERS:

To study the anticarcinogenic activity of Fenugreek seed extract, different parameters will be studied related to skin tumor model system. All the parameters will be studied after the autopsy of the animals. All the mice belonging to different groups will be sacrificed at the end of the experiment (i.e. 4 months).

C1) GENERAL PARAMETERS:

- Tumor incidence
- Tumor yield
- Diameter of tumor
- Tumor Burden
- Weight of tumor

C2) BIOCHEMICAL PARAMETERS:

The aim is to study the *in vivo* antioxidant activity of the seed extract. Enzymatic level in the liver of the mice will be studied. The liver will be immediately excised, washed with cold saline, blotted and a part of it will be minced and homogenized for:

NAME OF THE ENZYME	REFERENCES
Superoxide Dismutase (SOD)	(Marklund and Marklund, 1974)
Catalase(CAT)	(Aebi,1984)
Reduced Glutathione(GSH)	(Moron *et.al.,*1979)
Glutathione Peroxidase(GP_X)	(Paglia and Velentine,1967)
Glutathione S transferase(GST)	(Habig *et.al.,*1974)
Lipid Peroxidation(LPO)	(Ohkawa *et.al.,*1979)

C3) CYTOGENETIC STUDY:

The induction of genetic damage has clear and dramatic implications for human health, with teratogenic, mutagenic, and carcinogenic consequences resulting from cellular chromosomal alterations in appropriate tissues. To analyse the potential of an agent to initiate genetic damage a

variety of complementary assays may be employed. Cytogenetic assays have the important advantage that they enumerate damage at the level of the individual cell. Examples of the cytogenetic assays are the assays involving the examination of chromosomal aberrations at mitosis, of cells prior to mitosis using the technique of premature chromosome condensation, of micronuclei in post-mitotic cells and of sister chromatid exchanges. To observe the damage pattern two approaches will be used:

- Chromosomal Aberration Study(Tripathi *et al.,* 2011)
- Micronuclei Test (Heddle., 1973)

C4) MOLECULAR STUDY:

Stress stimuli such as DNA damage, hypoxia and oncogene activation p53, a tumor suppressor gene(TSG) gets activated. It plays an important role in determining cell's fate following DNA damage. p53 is regarded as a key factor in maintaining the balance between cell growth and cell death. Due to its role in regulation of cell cycle, alterations in p53 are critical events in carcinogenesis. Hence to understand the mechanism of tumor suppression, the effect of *Trigonella foenum graceum* will be studied either at the gene level or at the protein level.

For detection of the protein level of p53 study will be done by carrying out Western Blot by Towbin *et al.,*1979.

At the gene level genomic DNA will be isolated from blood. It will be then subjected to PCR amplification using primer for p53 gene (Ruggeri *et al.,* 1993).The oligonucleotide primer pair for PCR will be used is:

Sense: 5' GGG ACA GCC AAG TCT GTT ATG 3'

Antisense: 5' GGA GTC TTC CAG TGT GAT GAT 3'

(Kaur *et al.,* 2010)

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